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The Effects of Low Irradiance Long Duration Photochemical Internalization on Glioma Spheroids

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Abstract

BACKGROUND—Photodynamic therapy (PDT), if given over extended time periods (i.e. hours or days) and at very low irradiance in the μW/cm^2 range, has been shown to be more effective than acute PDT (aPDT) administered over minutes. This has led to the concept of metronomic PDT (mPDT), which consists of ultra-low irradiance light illumination for extended periods of time along with either continuous or repetitive delivery of photosensitizer.

Since the drug activating technology photochemical internalization (PCI) is based on PDT it seemed reasonable to expect that ultra-low irradiance, if administered over an extended period of time, could nevertheless result in effective metronomic PCI (mPCI) comparable to or more effective than that obtained with relatively high and short irradiance i.e. acute PCI (aPCI).

METHODS—Tumor spheroids consisting of F98 cells were used as *in-vitro* tumor models. The amphiphilic photosensitizer Al phthalocyanine disulfonate ($AlPcS_{2a}$) was used for all PCI experiments. Light treatment was administered from a diode laser at λ= 670 nm at various irradiance exposures of 2 mW/cm^2 for aPCI and 0.05 – 0.2 mW/cm^2 for mPCI with durations ranging from 3 – 12 min for aPCI and 120 minutes for mPCI.

RESULTS—$AlPcS_{2a}$ fluorescence was seen throughout the cytosol following short or long light treatment, corresponding to aPCI and mPCI respectively. Spheroid growth was significantly inhibited or completely suppressed at a mPCI radiance of 0.05 or 0.72 J/cm^2 respectively, with all bleomycin (BLM) concentrations used, compared to either BLM alone or aPCI at radiant exposure

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Compliance with ethical standards

Conflict of interest

All of the authors declare that she/he has no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

at these levels. The effects of BLM-aPCI and mPCI were comparable at radiance levels of 0.96 and 1.44J/cm^2.

CONCLUSIONS—Results show that mPCI could effectively cause significant spheroid growth inhibition with the delivery of extremely low light irradiance rates delivered over an extended period of time. These findings suggest that effective implementation of mPCI can deliver adequate drug efficacy at depths necessary to reach infiltrating glioma cells in the surgical resection cavity wall.

Keywords

photochemical internalization; PCI; metronomic photochemical internalization; mPCI; metronomic photodynamic therapy; mPDT

Introduction

Despite continued efforts, glioblastoma multiforme (GBM) remains an incurable form of primary brain cancer. Although the extent of surgical tumor resection is an important prognostic factor for patients with malignant gliomas, even in cases of gross tumor resection (as determined from post-operative MRI), the tumor invariably recurs. In approximately 80% of all cases, recurrent tumor growth occurs within a 2–3 cm margin of the surgical resection cavity [1, 2]. The therapeutic goal of post-operative therapy is therefore the elimination of infiltrating tumor cells remaining in the margins of the resection cavity. Presently, there is no single treatment modality that can accomplish this goal since infiltrating tumor cells can be found in the brain adjacent to tumor (BAT) region up to several cms from the resection site.

A number of light-mediated therapies, such as photodynamic therapy (PDT) and photochemical internalization (PCI), have been and are continuing to be developed for treating cancer. Photochemical internalization (PCI) is a novel technology that utilizes the principles of PDT to enhance the intracellular delivery of macromolecules in a site and temporal-specific manner. [3–7]. Macromolecules as well as some chemo-therapeutic agents that are internalized into cells via endocytosis, end up trapped in intracellular endosomes and lysosomes. The concept of PCI is based on using photosensitizers, which localize in the cell membrane and are carried into the cell covering the inner leaflet of the endosomal membranes. The photosensitizer remains in the endosome membrane while the macromolecule is localized within the lumen. Specific amphiphilic photosensitizers, like Al phthalocyanine disulfonate ($AlPcS_{2a}$) and meso-tetraphenyl chlorin disulphonate ($TPCS_{2a}$), preferentially accumulate in the membranes of endosomes and lysosomes [8, 9]. Upon light exposure, the photosensitizer interacts with ambient oxygen to produce singlet oxygen, which ruptures the vesicular membrane. The released agent can therefore exert its full biological activity, in contrast to being degraded by lysosomal hydrolases following endolysosomal fusion.

Light treatment for PCI is usually given over a period of minutes with moderate to high light irradiance, defined here as acute PCI (aPCI). Due to the rapid attenuation of light in tissue the ultra-low-intensity laser light irradiance, that would be found at depths in the BAT or in

extracranial tumors, would prove inadequate if delivered over these relatively short treatment periods [10, 11].

Photodynamic therapy (PDT) has been shown to be most effective at ultra low irradiance given over extended time periods (i.e. hours) [12–17]. This has led to the concept of metronomic PDT (mPDT) [18–22], which consists of irradiance light illumination for extended periods of time along with either continuous or repetitive delivery of photosensitizer. Several *in-vitro* and *in-vivo* studies, as well as a clinical study, have demonstrated the feasibility of mPDT or similar treatments (e.g. repetitive light illumination) in brain as well as other tumor forms. We hypothesized that the ultra-low-intensity light irradiance expected at depths in infiltrated tissue and tumors could nevertheless result in effective PCI mediated drug delivery and activation if administered over an extended period of time.

The aim of the present research was designed to demonstrate the ability of low light intensity PCI, termed metronomic PCI (mPCI) to enhance the effectiveness of chemotherapy on multicell 3-dimensional glioma tumor spheroids. In contrast to cell monolayers, tumor spheroids mimic *in-vivo* tumors in their micro-environment in terms of gene expression, and in particular oxygen gradient characteristic and the biological behavior of the cells growing in three dimensions.

Materials and Methods

Cell Lines and Drug

The F98 rat glioma cells were obtained from the American Type Culture Collection (Manassas, VA). The cell line was maintained in Advanced DMEM medium (Thermo Fisher Scientific, Carlsbad, CA) supplemented with 2% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES buffer, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C, 5% CO_2 and 95% humidity. The F98 cells were grown as monolayers in T-25 tissue culture flasks, (Greiner BioOne Frickenhausen Germany). Bleomycin (BLM) was obtained from Sigma Aldrich (St. Louis, MO).

Localization and Redistribution of $AlPcS_{2a}$

10×10^3 F98 cells were plated out in glass bottomed imaging dishes (FluoroDish, World Precision Instruments, Sarasota, Florida) for 48 hrs. 1 μg/mL $AlPcS_{2a}$ was added and the cells were incubated for an additional 24 hours. The monolayers were washed twice and allowed to soak in photosensitizer-free medium for four hours prior to light treatment. Images were visualized using an inverted Zeiss laser-scanning microscope (LSM, Carl Zeiss, Jena, Germany).

aPCI/mPCI Treatment of Glioma Spheroids

The aPCI and mPCI protocol used is shown in figure 1. F98 spheroids were formed by a modification of the centrifugation method previously described [23]. F98 glioma spheroids were generated with 2.5×10^3 cells in 200 μL of culture medium per well of an ultra-low attachment surface 96-well round-bottomed plate. Immediately following centrifugation, the

tumor cells formed into a disk shape. The plates were maintained at 37°C in a 5% CO_2 incubator for 24 hrs. to allow them to take on the usual three-dimensional spheroid form. Twenty four hours after spheroid formation 0.5 μg/mL photosensitizer ($AlPcS_{2a}$; Frontier Scientific Inc., Logan, UT) was added to the wells for an additional 18 hrs. at 37°C and 5% CO_2. Following incubation, spheroids were washed 4 times in the plates. For PCI, the spheroids were incubated in the presence of BLM for an additional 4 hours.

Light treatment was administered from a diode laser at $\lambda = 670$ nm at various irradiance exposures of 2 mW/cm^2 for aPCI and 0.05 – 0.2 mW/cm^2 for mPCI with durations ranging from 3–12 min for aPCI and 120 minutes for mPCI.

All laser irradiation was done from underneath the plate so the beam passes through the bottom of the well, directly on the spheroids. Light absorption by remaining BLM or the phenol red in the culture medium is therefore not a factor.

Typically, 16 spheroids for the various groups were followed in 3 individual trials for up to 14 days of incubation. Culture medium in the wells was exchanged every third day. Determination of spheroid size was carried out by measurement of their diameter using a microscope with a calibrated eyepiece micrometer and their volume calculated assuming a perfect sphere. Control cultures received light treatment but no BLM (PDT control) or BLM but no illumination (drug only control). Following PCI, the plates were returned to the incubator and monitored for growth over a 14 day incubation interval.

Statistical Analysis

Microsoft Excel was used to determine the mean and standard deviation. Data were analyzed using one-way ANOVA at the significance level of $p<0.05$ and presented as mean with standard deviation, unless otherwise noted.

Results and Discussion

BLM has previously been used in a number of PCI experiments [23 – 27] and was therefore chosen for this study. In order to determine the cytotoxic effects of low irradiance BLM-PCI on F98 spheroids it was necessary to establish a suboptimal BLM dose to be used in subsequent experiments. F98 spheroids were exposed to BLM over a concentration range of 0.15 – 2.4 μg/mL (figure 2a). Since 0.15 μg/mL of BLM gave no significant effect and 2.4 μg/mL was highly toxic, the concentration range of 0.3 – 1.2 mg/mL was used in all subsequent experiments. BLM, a large (MW: 1.5kDa) water-soluble glycopeptide, enters cells via endocytosis [28]. BLM is approved for a number of standard cancer chemotherapy treatments, such as head and neck tumors, lymphoma and testicular cancer, but due to its limited ability to escape endolysosomal entrapment and degradation, high systemic doses are required. This low therapeutic window can lead to unacceptable side effects, the most serious being lung fibrosis [29]. However, if released from entrapment, for example by PCI, it quickly diffuses into the nucleus where it has a significant toxic effect causing both double and single DNA strand breaks [30, 31]. It has been estimated that as little as five hundred bleomycin molecules translocated to the cytosol may be sufficient to kill a variety of cancer cells [32].

To determine the light irradiance range for mPCI (i.e. PDT response), spheroids were irradiated with λ = 670 nm at increasing radiant exposures ranging from 0 – 1.44 J/cm^2 at an irradiance of 100 μW/cm^2 (figure 2b) with an irradiation time of 120 minutes. Although PCI is usually most effective with a spheroid PDT survival of approximately 80% [24], both the aPCI and mPCI experiments were also performed in a light irradiance range of 0 – 1.44 J/cm^2 for a more complete demonstration of the differences between the two protocols.

Endosomal Escape of AlPcS$_{2a}$ After Light Irradiation

Fluorescence microscopy was used to verify the uptake, intracellular localization and redistribution of the photosensitizer AlPcS$_{2a}$ in the absence or presence of light (figure 3). 10×10^3 F98 cells were plated out in glass bottomed imaging dishes for 48 hrs. AlPcS$_{2a}$ of 1 μg/mL was added and the cells were incubated for an additional 24 hours. The monolayers were washed twice and allowed to soak in pure medium for four hours prior to light treatment. Light treatment was administered over a 6, 60 or 120 minute period at 2 or 0.1 mW/cm^2 respectively. As seen in figure 3a, the photosensitizer (red) was taken up in the F98 dark control cells and localized in granular organelles representing endosomes and lysosomes, as previously observed for other cell types [6]. One hour post light treatment of either a short (6 min) or long (120 min) exposure times, a similar diffuse AlPcS$_{2a}$ fluorescence throughout the cytosol (excluding the nucleus, figure 3b and d) was observed for both irradiances exposures, indicating a light-induced endosomal membrane release of the photosensitizer. As seen in figure 3c, a radiant exposure of 60 minutes, corresponding to 0.36 J/cm^2, revealed cells where the photosensitizer could be found either still bound in endosomes or diffused throughout the cell cytosol. This would indicate an intermediate state in an ongoing photochemical process or a suboptimal light dose for lysosomal rupture.

AlPcS2a, in common with other efficient PCI photosensitizers, is an amphiphilic photosensitizer which binds to the plasma membrane and localizes to the membranes of endocytic vesicles after endocytosis (figure 3a) and as previously reported [3, 8, 24].

Light activation of the photosensitizer, at low irradiance, induces rupture of the lipid layer of the endolysosomal membranes, followed by the release of the photosensitizer into the cytosol as shown in figure 3b, c and d, and as previously shown for aPCI [4].

Comparison of the Effects of BLM-aPCI or BLM-mPCI on Spheroid Growth

The comparative effects of BLM-aPCI and mPCI on F98 glioma spheroid growth is shown in figure 4a – d at four increasing radiance levels. aPCI was delivered at an irradiance of 2 mW/cm^2 for exposure of 3, 6, 8 or 12 minutes, corresponding to a radiant exposure range of 0.36 to 1.44 J/cm^2. At a radiant exposure of 0.36 or 0.72J/cm^2, aPCI showed a relatively modest increase of BLM efficacy compared to drug alone. In contrast, at a radiant exposure of 0.96 J/cm^2 and 1.44 J/cm^2, a pronounced and significant inhibition of spheroid growth was demonstrated for all of the BLM concentrations used.

BLM-mPCI was carried out at irradiances 0.05, 0.1, 0.133 and 0.2 mW/cm^2, to simulate light levels encountered at differing tissue depths and BLM concentrations of 0.3, 0.6 and 1.2 μg/mL. Light irradiance exposure was held constant at 120 minutes corresponding to radiant exposures of 0.36, 0.72, 0.96 or 1.44 J/cm^2 respectively as was the case for aPCI. At

the two lowest radiance levels (0.36 and 0.72J /cm^2) significant growth inhibition ($p<0.05$), compared to that obtained for aBLM-PCI, could be demonstrated (fig 4a and b). The difference was especially pronounced at a radiance level of 0.72 J/cm^2 (fig 4b). At the two higher levels of radiance (0.96 and 1.44 J/cm^2) the inhibitory effects seen for either protocol were similar and the difference only significant ($p<0.05$) at the lowest BLM concentration (Fig 4c).

In contrast to the results shown in figure 4, no significant BLM-mPCI spheroid growth inhibition was produced at an irradiance of 20 μW/cm^2 with light application over a 20 hour time period, corresponding to a radiant exposure of 1.44 J/cm^2 (data not shown). This would indicate that minimum threshold irradiance is necessary for efficient mPCI regardless of the total light radiance delivered.

The therapeutic effects of PCI treatment at light radiance levels up to 0.96 J/cm^2 appear to be primarily due to the drug cytotoxicity and only to a minor degree due to PDT. This is demonstrated in figure 4 a–c, which clearly illustrates PCI's ability to utilize low doses of light and BLM, while at the same time maintaining a high tumor cytotoxic effect as has also been demonstrated in a host of previous publications [6].

The kinetics of spheroid growth for three increasing irradiation is shown in figure 5a, b and c. Light was administered over 120 minutes. The BLM concentration used in the experiments shown in figure 5 was 0.6 μg/cm^2. BLM-mPCI significantly inhibited spheroid growth at an irradiance of 0.05 mW/cm^2 compared to BLM or PDT treated cultures acting singularly (figure 5a). Spheroid growth was completely suppressed by BLM-mPCI at both 0.1 and 0.133 mW/cm^2. PDT at these irradiations demonstrated growth delay, but by day 14 the spheroid volumes were not significantly different from untreated controls.

The basic underlying mechanism for PCI, controlled endolysosomal escape of therapeutic molecules, has been verified in a large number of experimental studies. When first developed PCI protocols employed the so called "light-after" approach since it was thought that the photosensitizer and the compound to be released from the endocytic vesicles had to be located in the same compartments at the time of light exposure [3–5]. However, follow-up studies have shown that light can be delivered prior to the delivery of the compound without reducing the efficacy of the combined treatment [33, 34]. In the BLM-mPCI experiments reported here, light was administered over a period of hours and BLM was present before, during and after light irradiation. In this case both the light after and light before strategies are activated. Inside a cell, a large number of physical and biochemical effects are occurring simultaneously in an ongoing dynamic fashion. The actual time course kinetics of the continuous drug endocytosis, endosome, lysosome formation, endo-lysosome fusion and drug degradation is presently unknown in detail. One explanation for the results seen with mPCI could be the continuous induced escape of BLM by PDT effects during the long duration light exposure. This would be in contrast to the relative "snap-shot" light exposures generally used in aPCI. PCI-induced release of macromolecules has been shown to occur within a range of 0.01 – 4 s depending on the nature of the released agent [35].

Both the aPCI and mPCI protocols used in these experiments delivered equivalent light radiance levels over the course of treatment. It is highly probable that they share a similar mechanism of action, that is, the PDT mediated generation of singlet molecular oxygen leading to rupture of endosome and lysosome membranes. The similar redistribution of photosensitizer following either short, intermediate or long duration light treatment, shown in figure 3, would support this explanation.

Theoretical modeling in multicell spheroids suggests that the spatial distribution of singlet oxygen (the primary cytotoxic agent in PDT) depends on the irradiance and on the availability of ambient oxygen [36]. High irradiance consumes and rapidly outstrips the diffusion of oxygen throughout the spheroid. In contrast, very low irradiance delivered over longer periods, would allow for a better availability of oxygen diffusing throughout the spheroid. Our results agree well with the recent mPDT in vivo brain tumor study of Guoa et al. Although not a PCI study, they could demonstrate a single dose of ALA and a light exposure of 3.7 hours, considered there as mPDT, resulted in increased survival compared to control animals [22]. The light exposure duration used in the above study is comparable to the one used in the mPCI experiments reported here.

Since the optical penetration depth in human brain at $\lambda = 670$ nm is around 0.4 cm, light at this wavelength is reduced by a factor of 1000 in approximately 2.8 cm of brain tissue. A surface irradiance of 50 mW/cm^2 would result in an irradiance of 50 $\mu W/cm^2$ at this depth, an irradiance capable of inducing effective BLM-mPCI (figure 4 and 5). From a clinical perspective, these calculations suggest that irradiance rates adequate for effective mPCI can be delivered to depths exceeding 2 cm in the post-operative resection margin. Norum et al. has reported that BLM-aPCI has been shown to be effective in sterilizing the tumor bed after marginal cytoreductive surgery using an invasive human fibrosarcoma in a mouse model [26]. Further validation of the mPCI effect to inhibit tumor recurrence following cytoreductive surgery needs to be carried out using relevant *in-vivo* brain tumor models.

Conclusion

The key findings of this study were that BLM-mPCI, employing extremely low light irradiance in the $\mu W/cm^2$ range delivered over an extended time period, resulted in significant spheroid growth inhibition. The experimental results suggest increased effectiveness of mPCI, with ultra-low light levels over a longer treatment duration, compared with that of the standard aPCI technique at higher irradiances over a much shorter duration of light treatment. The redistribution of the photosensitizer from endosomal entrapment to the cell cytosol localization by light exposure was equivalent for either aPCI or mPCI.

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Highlights

- BLM-mPCI delivered over an extended period of time resulted in significant spheroid growth inhibition.
- Metronomic PCI (mPCI) had increased effectiveness compared to the standard acute PCI (aPCI) technique.
- The photosensitizer redistribution from endosomal entrapment to cytosol localization following light exposure was equivalent for either aPCI or mPCI.

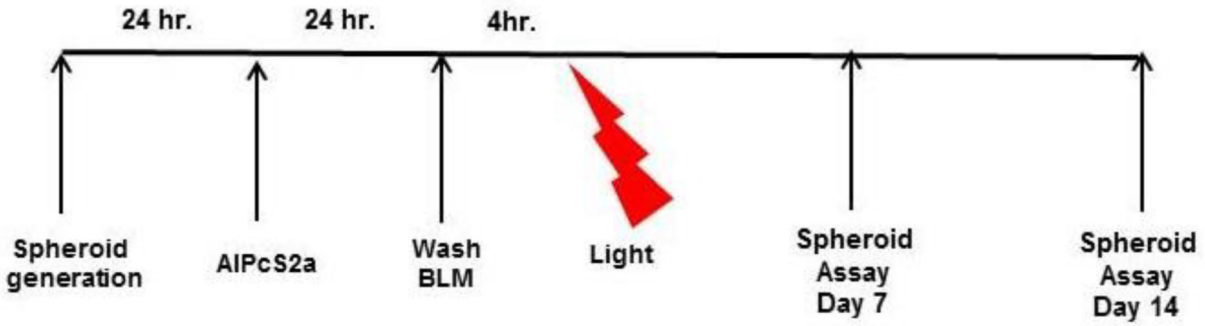

Figure 1.

Experimental protocol for aPCI and mPCI. F98 spheroids were generated and incubated for 24 hours. Photosensitizer ($AlPcS_{2a}$) was then added. After incubating for an additional 24 hours, spheroids were washed and BLM at various concentrated added to the culture medium. Four hours later light treatment was initiated for aPCI (3 – 12 min @2mW/cm^2) or mPCI (120 min @ 0.05, 0.1, 0.133 or 2mW/cm^2). The spheroids were then assayed on day 7 and day 14.

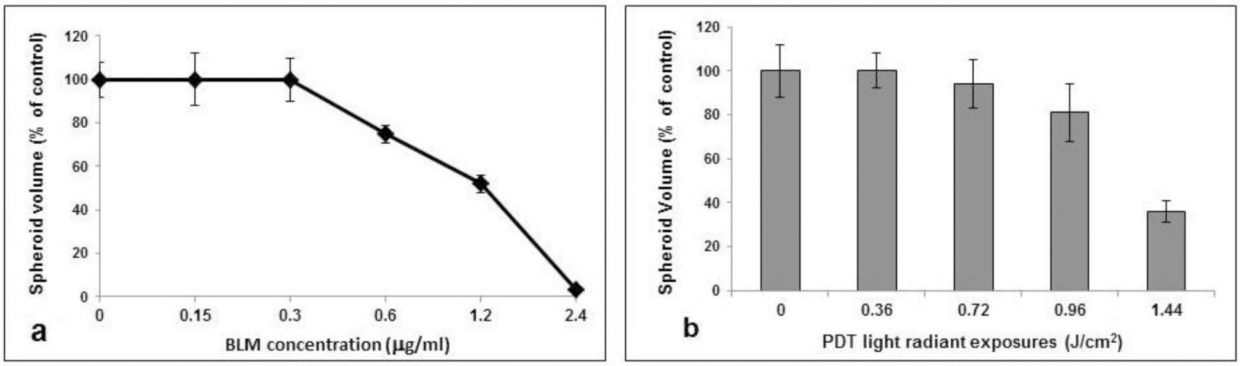

Figure 2.

Cytotoxic effect of BLM alone and PDT alone on F98 tumor spheroids. (a) Spheroids were treated with BLM at varying concentrations (0 – 2.4 μg/mL). Cell viability was assessed by measuring spheroid volume at 7 days and 14 days. (b) Spheroids were irradiated with λ = 670 nm at radiant exposures ranging from 0–1.44 J/cm^2 at an irradiance of 0.1 mW/cm^2 for up to 240 minutes. Each data point represents mean ± standard deviation of eight trials.

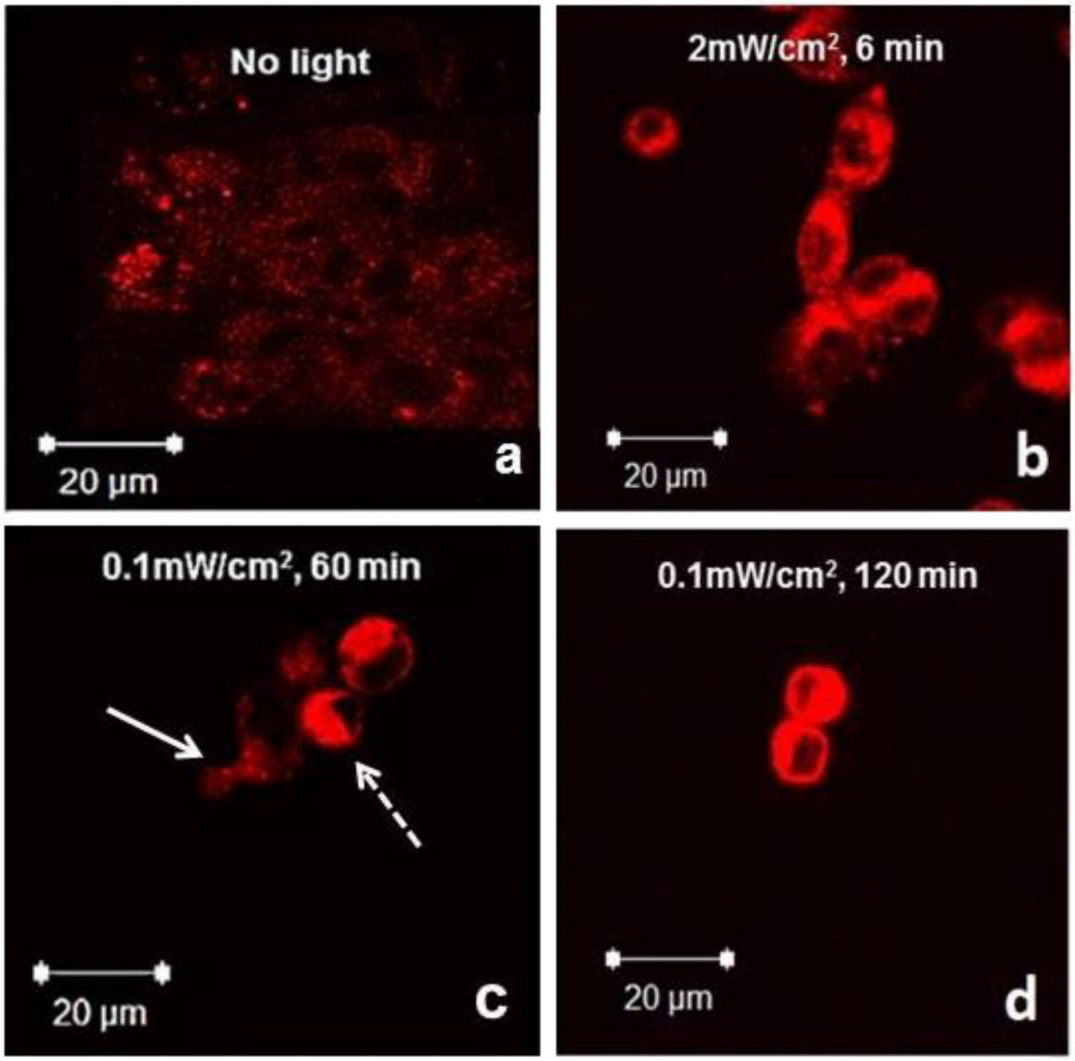

Figure 3.

Visualization of $AlPcS_{2a}$ with endosomal escape at varying radiant exposures. F98 cells were incubated with 1 μg/mL $AlPcS_{2a}$ for 24 hours, washed twice, and allowed to soak for four hours prior to visualization. Images were captured with fluorescence microscopy following light treatment at: (a) No light, (b) light, 6 minutes at 2 mW/cm^2, (c) light, 60 minutes at 0.1 mW/cm^2, solid arrow cells where photosensitizer is still bound to endosomes, dotted arrow cells with escaped photosensitizer, and (d) 120 minutes at 0.1 mW/cm^2. $AlPcS_{2a}$ is shown by red fluorescence dispersed throughout the cell cytosol. Scale bar represents 20 μm for all images.

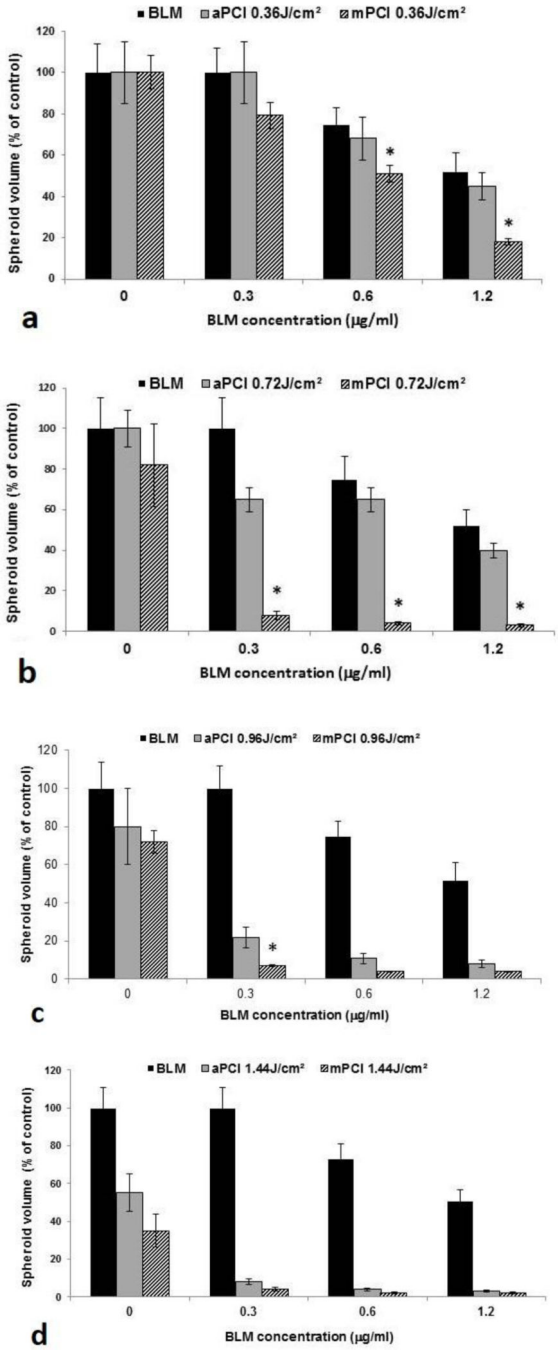

Figure 4.

Comparison of the effect of BLM-aPCI and BLM-mPCI on growth of F98 spheroids. BLM concentration from 0–1.2 µg/mL for both protocols. Irradiance for BLM-aPCI of 2mW/cm^2 delivered for (a) 3, (b) 6, (c) 8 and (d) 12 minutes. Irradiance for BLM-mPCI of (a) 0.05, (b) 0.1, (c) 0.133 and (d) 0.2 mW/cm^2 with exposure time of 120 minutes for all cases. Corresponding radiant exposure of (a) 0.36, (b) 0.72, (c) 0.96 or (d) 1.44 J/cm^2, respectively for both protocols. Each data point represents mean volume of 16 spheroids after 2 weeks in

culture as a % of non-treated controls. Error bars; standard deviation. *Represents significant differences ($p<0.05$) between aPCI and mPCI treatment.

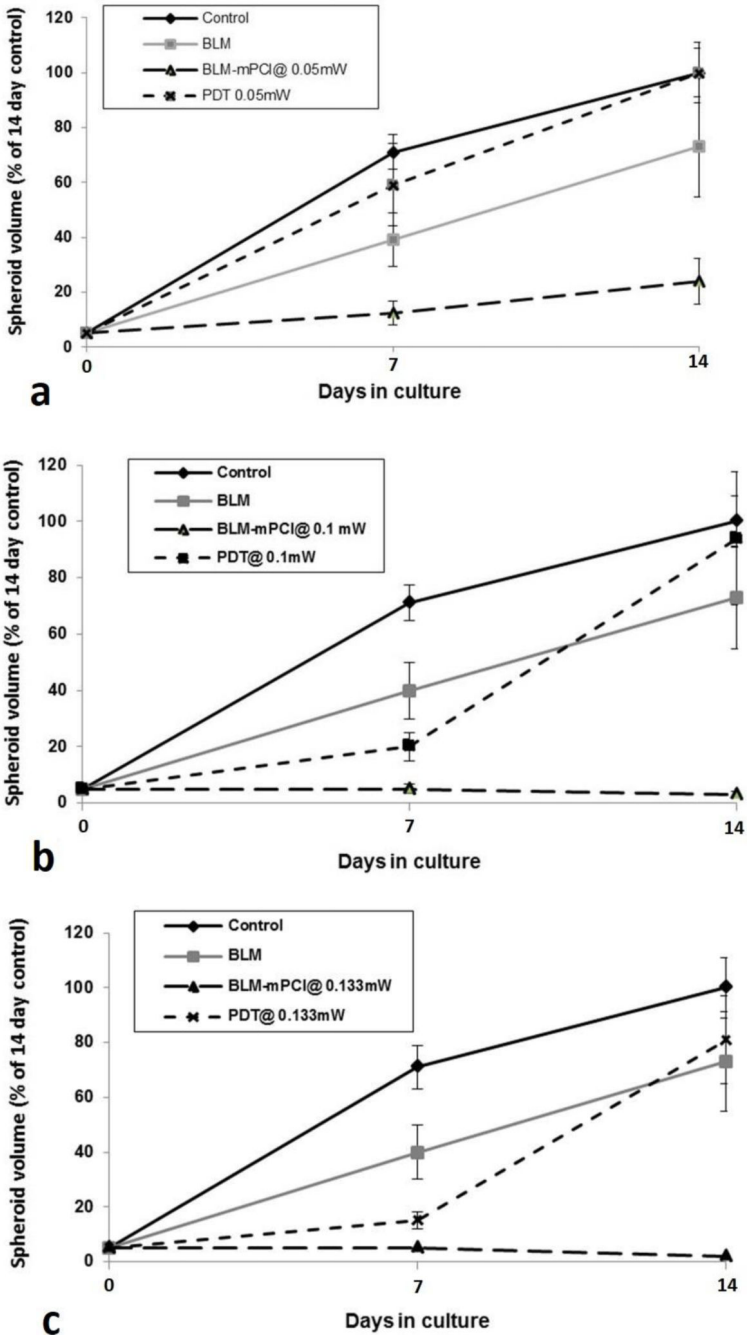

Figure 5.

Kinetics comparison of F98 spheroid growth with BLM alone, BLM-mPCI and PDT at varying irradiation rates. Light administered over 120 minutes. BLM concentration was 0.6 μg/cm^2. BLM-mPCI and PDT at irradiance levels of (a) 0.05, (b) 0.1, and (c) 0.133 mW/cm^2, corresponding radiant exposure of 0.36, 0.72 and 0.96 J/cm^2 Each data point represents mean volume of 16 spheroids from 2 experiments after 14 days in culture as a % of non-treated control spheroid volume on day 14. Error bars; standard deviation.